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The physiological status of isolated oat leaf protoplasts.

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THE PHYSIOLOGICAL STATUS OF ISOLATED OAT LEAF PROTOPLASTS

A Thesis Presented

by

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PREFACE

This thesis is composed of three parts:

Part I is a literature review in the form of a paper to be submitted to Botanical Reviews.

Part II deals with my research on isolated oat leaf protoplasts and take the form of a paper to be submitted to Plant Science Letters.

Part III is an appendix containing a record of some experiments and material which will not be published.

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PART I

LITERATURE REVIEW

PLANT CELL PROTOPLASTS:

ISOLATION AND USE FOR PHYSIOLOGICAL STUDIES

ABSTRACT

Much attention has been focused on the use of protoplasts for the culture, somatic cell fusion, and genetic alteration of higher plants. However, the growing body of literature concerned with the use of protoplasts for the study of plant cell physiology has not been reviewed.

Basic techniques for protoplast isolation and purification are discussed with physiological interest in mind. We reviewed physiological studies including studies of membrane binding and transport, the action of growth regulators, especially the auxins, studies on photosynthesis, respiration and nucleic acid metabolism. In addition, the use of protoplasts to obtain cellular organelles is discussed.

RÉSUMÉ

On a foyé beaucoup d'attention sur l'emploi des protoplastes en culture, la fusion des cellules somatiques, et les altérations genetiques des plantes superieures. Cependant, la litterature qui s'agit de l'emploi des protoplastes pour l'étude physiologique des cellules des plantes n'était pas examinée.

Les techniques fundamentales pour l'isolement et la purification sont disuctes avec réference spéciale a l'interêt physiologique: l'attachement des substances á la membrane et le transport par la membrane, l'action des hormones, surtout les auxines, les études de la photosynthese, de la respiration, et de la metabolisme des acides nucleiques. Aussi, l'emploi des protoplastes pour obtenir les organelles cellulaires est disucté.

II. Introduction

Protoplasts provide a potentially powerful tool for plant breeders interested in somatic cell fusion and regeneration of genetically modified plants (19, 27, 94). The concept of using protoplasts for physiological studies has, in part, originated as a peripheral interest by workers anxious to demonstrate that their experimental material is physiologically healthy. The use of protoplasts to study plant cell physiology has developed more recently.

Protoplasts provide an excellent material to explore such areas as the transport of metabolities and ions into plants (76, 81, 92), the uptake of virus particles (91, 101), and the interaction of auxins with the plasmalemma (5, 42, 65, 66, 87). Such studies were heretofore limited by the use of intact tissue where results were confounded by possible binding of materials in the intercellular spaces, the probability that not all cells are equally exposed to substrate due to the necessity of diffusion through cell layers, and the possible transport of materials back into the bathing medium via vascular tissue when excised tissues are used (50). Such problems may be partially overcome by the use of protoplasts as the absence of the cell wall eliminates some possible binding sites, the removal of vascular strands with the tissue debris eliminates their participation in uptake and transport, and, since one is working with a fairly homogeneous suspension of cells, equal exposure of all cells to substrate is achieved.

Additional advantages for the use of protoplasts can be found in metabolic research. For example, the ability to separate mesophyll cell protoplasts from bundle sheath strands in plants exhibiting the C_4 dicarboxylic acid pathway of photosynthesis (39, 48, 54) provides the researcher with the ability to study the individual contributions of mesophyll and bundle sheath cells. This approach eliminates the problem of cross contamination of enzymes and metabolities incurred during the sequential grinding techniques previously used (10). Protoplasts have also been used to obtain cellular organelles such as chloroplasts (73) and nuclei (7) which posess a higher degree of integrity than those obtained by grinding intact tissue.

The purpose of this review is to provide a comprehensive treatment of techniques for the isolation and purification of protoplasts for physiological studies, and to review the physiological work which has been done. Extensive reviews of the literature concerning the use of protoplasts for genetic study and somatic cell breeding can be found elsewhere (19, 27, 94). That literature, therefore, will not be considered here.

III. Techniques.

A. Protoplast Isolation.

1. Mechanical Techniques. A preparation of numerous protoplasts was first reported by Klerker in 1892 (27, 94). Klerker and his contemporaries isolated protoplasts exclusively by mechanical methods, which involved plasmolysis of the tissue such that the cell wall was cut without damage to the protoplast. The protoplasts were released

by slight deplasmolysis (27), or by gentle teasing with a micropipette or needle (16). These procedures are rarely used now as the yields are considerably lower than with the enzymatic procedures currently available, many cells are damaged, and the procedures are largely restricted to parenchymatous cells of storage tissue. Such tissues include those of onion and beet, where there are highly vacuolated cells that contract from the cell wall completely upon plasmolysis (27, 94).

2. Enzymatic Techniques. The earliest reported use of enzymes to obtain numerous protoplasts was by Cocking (18) who isolated protoplasts from root tips of tomato seedlings with a crude cellulase preparation from Myrothecium verrucarria. The use of pectolytic and/or celluolytic enzymes permit the isolation of protoplasts from many sorts of plant tissues including leaves (9, 26, 54, 90, 92, 99), roots (18, 66), root nodules (22), fruit (36, 72), aleurone layers (89), coleoptiles (41, 78), callus cultures (3, 61), and cell suspensions originating from callus culture (30, 25, 83).

Cellulases and petinases became available commercially in the middle of the 1960's. Cellulase Onozuka, still extensively used, was first available at that time, and since then many other preparations have come onto the market (79). While the commercial enzymes are convenient to obtain and lead to high reproducibility of protoplast preparations, a few workers prefer to purify their own enzyme preparations contending that they are more effective (77). Others have made claims that the commercial preparations are contaminated with nucleases

(especially ribonuclease) (57, 94), proteases, lipases, phenolics, and other toxic substances (94). As a result of these reports, some workers have resorted to putting the enzymes through Sephadex or Biogel (56, 83) which results in the removal of salts, phenolics and other toxins but not contaminating enzymes. It is possible to obtain a highly purified cellulase, but the purification process is laborious. There is also a loss of effectiveness in protoplast production, for both detrimental enzymes and possible helpful "contaminants", such as hemicellulases and pectinases are removed during purification (94). A pre-plasmolysis period is another method used to overcome the more adverse effects of contaminants. Withers and Cocking (100) showed that invagination of the plasmalemma, resulting in the formation of internal vesicles, occured during the process of plasmolysis. If a marker, such as thorian dioxide, was included when the cells were first plasmolysed, it was found in the intracellular vesicles, but if it was not added until the initial plasmolysis was complete, a much smaller quantity was in the vesicle. Pre-plasmolysis, therefore, can be used to avoid the uptake of cellulase and attending contaminants into the cell.

Procedures using cell wall degrading enzymes vary greatly. The classic technique for the two-stage isolation procedure was first introduced by Takebe et al. (90). Tobacco leaves with the epidermis peeled are first treated with pectinase. The isolated cells resulting from this treatment can then be made into protoplasts through cell wall removal by cellulase. Power and Cocking (70), also using tobacco leaves, varied the technique by using cellulase and pectinase together.

This basic technique of employing enzyme mixtures has been applied to the isolation of protoplasts from many different species and tissues (48, 54). In some cases, however, it has been found that the pectinase is unnecessary and a cellulase preparation alone has been used (9, 48). In rare cases, such as the isolation of protoplasts from pollen tetrads (6) or aleurone layers (89), an additional enzyme which is isolated from snail guts and rich in β-1,3-glucanase is necessary to break down these specialized cell walls.

Since enzyme mixtures cannot penetrate the cuticle, the tissues must be prepared in a number of ways before subjecting them to digestion. The options include slicing it into small pieces (53, 54), removing the lower epidermis (90, 92), or peeling away the cuticle (29).

Various concentrations of plasmolyticum have been employed in the preparation of protoplasts. Shepard and Totten (86) reported an isolation procedure in which a non-plasmolysing concentration of sucrose (0.2M) was used. However, the majority of preparations call for osmotic concentrations ranging from 0.6M (30, 54, 92) to 0.8M (90), and in one case 0.9M (11). Initially, especially with the mechanical isolation procedures (16, 84), ionic salts were used to make up the osmotic concentration. With the advent of the enzymatic isolations, Cocking (18) introduced the use of sucrose which, in addition to providing an osmoticum, has the advantage of being dense enough such that the protoplasts float to the top for collection. Currently, however, mannitol and sorbitol are being used more extensively as sucrose has been found to be harmful to the protoplasts. Fodil et al. (28) found that Avena coleoptile protoplasts were unstable and tended to extrude their

protoplasm when isolated in sucrose. Also with Avena coleoptile protoplasts, Ruesink and Thimann (78) found inhibition of digestion when sucrose was used. Gambrow et al. found that the division of protoplasts from carrot suspension culture was delayed 1-2 days and was reduced by 20-50% when 0.05% sucrose was used in the incubation medium rather than glucose (30).

Other substances besides the basic osmoticum are often added to the digestion medium. While entirely ionic osmotica were discarded because they resulted in the external environment of the cell being charged in excess of the physiological level, Ruesink and Thimann were able to achieve higher yields of protoplasts in an entirely ionic osmoticum than with mannitol alone (78). They, therefore, suggested the inclusion of ions in a sorbitol or mannitol medium. Divalent cations were especially recommended since there is evidence that they play a role in maintaining membrane stability and function whether intact excised tissue (24) or protoplasts (37) are involved. This effect may involve increased fluidity of the membrane due to a localized alteration of the ordered-to-fluid phase transition temperature (93). Calcium has been found to be the most effective of the "membrane stabilizing" divalent cations with regards to both artificial lipid bilayers (93) and biological membranes (24, 78). High molecular weight substances such as potassium dextran sulfate or polyvinylpyrrolidone (PVP) are occasionally included. Takebe et al. reported enhanced protoplast production and viability with potassium dextran sulfate in the digestion medium. They postulated that the potassium dextran sulfate binds to some basic protein present as a contaminant in the enzyme mixture and suggested that

ribonuclease as a probable candidate (90). Ruesnink and Thimann (78) have shown ribonuclease to be more effective in the disruption of protoplasts than lipases or proteases (see section IV 3). PVP has been shown, in some cases, to increase protoplast production (86). This enhancement is thought to be the result of the PVP binding of toxic polyphenols which may be present in the enzyme preparation (86) or released by the plant tissue during digestion.

B. Protoplast Purification.

There are numerous ways to collect and purify protoplasts once they are released from the tissue. Protoplasts isolated in sucrose (20%) will float to the top of the solution and can be collected. Resuspension in a fresh solution of 20% sucrose and subsequent floatation constitutes a washing procedure (18). An interesting variation of this technique was utilized by Chaupeau and Morel (17) who, in order to purify carrot protoplasts, made a discontinuous gradient. They were able to maintain the osmotic concentration while increasing the density by changing the relative proportion of sorbitol and sucrose. The protoplasts collected just above the lowest layer (20% sucrose), while the debris collected at various other interfaces.

When mannitol or sorbitol are used as the osmotic stabilizers, the protoplasts are generally collected by a low speed centrifugation (50-100 x g), which may be preceeded by straining through a fine mesh seive (29, 54, 64, 92). Centrifugation accelerated the natural tendency of the protoplasts to settle out of the less dense mannitol or sorbitol solutions (3, 79, 89). While such centrifugation and repeated washes lead to the removal of some cellular debris and residual cellulase (9),

further purification is often desirable. The use of two-phase polymer gradients has recently been described as a procedure to separate particles of divergent sizes (1). Kanai and Edwards (55) applied this system to the problem of protoplast purification using a polyethylene glycol-dextran gradient. Centrifugation of the components resulted in the formation of two phases. The nature of these systems is such that the majority of the larger particles, i.e. protoplasts, should move to the interface and the smaller debris, i.e. chloroplasts, mitochondria, membrane particles etc. would tend to be found in the lower phase. Others (3, 92) have purified protoplasts by the simpler technique of allowing the protoplasts to settle out of solution one or more times, followed by removal of the supernatant containing the debris.

IV. Physiological Studies with Protoplasts

A. Membrane Studies.

Protoplasts provide a promising system for the study of the behavior of the plasma membrane, since in the absence of the cell wall one is working with an apparently homogeneous suspension. This presumably eliminates the problem of diffusion, as the plasmalemma of each individual cell is directly exposed to the substrate to be taken up. In addition, uptake is not confounded by the possible movement of substrate into conducting tissue.

1. Binding and Uptake of Large Particles. Protoplasts have been used in a number of interesting ultrastructural studies of binding of substances to the plasmalemma. Power and Cocking (70) were able to show binding to the membrane and subsequent pinocytotic uptake of

ferretin by tobacco protoplasts. Pinocytotic uptake has been offered as an explanation for the uptake of macromolecules i.e. RNA from virus (2), exogeneous DNA (44, 63), protein (43), as well as intact virus particles (91). However, chloroplasts (8, 15), nuclei (69), and bacteria (21) appear to be taken up by other means. Burgess et al. put forth some serious objections to attributing the uptake of large particles to pinocytosis (12). The protoplasts in which pinocytotic uptake was first observed degenerated after only eighty hours in culture, therefore, they may not have been physiologically sound enough to prevent penetration by large foreign particles. In addition, poly-L-ornithine which is used in animal systems to stimulate naturally occurring pinocytosis by altering membrane charge, is often required for this sort of uptake (91). At this time it is not known to what extent pinocytosis occurs in plant cells (12, 23). Poly-L-ornithine is known to damage the plasmalemma at high concentrations and it is not known whether the 1-2 μg/ml used constitutes a "high concentration" for plant cells. The increase of cellular debris in protoplast preparations exposed to this concentration indicates that it may be too high. The observed pinocytosis, therefore, may not be a naturally occurring process but the result of membrane damage.

Recently, Suzuki et al. (88) reinvestigated the uptake of polystyrene spheres and presented ultrastructural and biochemical evidence for pinocytosis. Poly-L-ornithine increased adsorption of spheres to protoplasts and, thereby, increased uptake. There was no evidence of damage to the membrane and the formation of vesicles by invagination

was clearly shown. In addition, the uptake was inhibited by sodium azide and dinitrophenol, suggesting a dependence on oxidative pathways of metabolism.

Burgess and Linstead (13) showed that ferretin labeled concanavalin A bound to the plasmalemma with different patterns, depending on whether protoplasts from tobacco leaf or grape vine suspension cultures were used. Concanavalin A is a phytohemagglutinin or lectin which binds to α-D-glucopyranosides and other stearically related sugars (33). Burgess and Linstead, therefore, concluded that there were different locations of sugar residues with respect to the membranes of the different protoplasts, but were unable to distinguish if this was due to different glycoprotein binding sites on the membrane, or nascent cell wall material on the tobacco protoplasts which are capable of regenerating a cell wall.

Differences in the plasma membrane have also been shown with regards to pinocytotic uptake of polystyrene latex beads by Mayo and Cocking (60). They were able to show denser phosphotungstic acid staining in the areas of the plasmalemma with higher pinocytotic activity and postulated that high levels of hydroxyl groups may be concentrated in these areas. However, Suzuki et al. also using polystyrene latex spheres, found random uptake.

Protoplasts can be induced to take up particles as large as bacteria. The rationale for research on the uptake of bacteria assumes that endosymbiotic relationships similar to the postulated symbiotic origin of chloroplasts and mitochondria (59) may still be established.

If so, the application becomes clear, protoplasts can be induced to take up nitrogen fixing bacteria such as Rhizobium sp. with the hope that as the protoplasts divide in culture, the bacteria will divide synchronously and maintain their ability to fix nitrogen in their new host (21). Another possibility, however, exists for the establishment of nitrogen fixation in non-leguminous plants. The fusion of isolated root nodule protoplasts (22) with protoplasts from a non-legume and subsequent regeneration of the hybrid is an exciting possibility.

Protoplasts have been shown to take up viral RNA (2) and intact viruses, and even the production of progeny virus has been demonstrated (91). Protoplasts posess a number of advantages for the study of viral infection in that one is working with a homogeneous suspension which enables the induction of synchronous infection. In addition, the level of infection is ten-fold greater (91, 101) and changes at the cellular level can be observed earlier than when one is limited to searching for symptomatic indications of infection in intact tissue (101). However, it should be remembered that poly-L-ornithine is required for the uptake of virus by protoplasts (91) and since there is some controversy surrounding its use (12, 88) caution should be exercised in the interpretation of these experiments with relation to the mode of viral attack.

2. Transport of Metabolites. Little work has been done with protoplasts concerning the uptake of small substances i.e., mineral ions and metabolites, in spite of the fact that protoplasts appear to be an excellent system to study these membrane processes. Taylor and Hall (92) studied the uptake of ^{86}Rb by maize leaf protoplats. They

were able to show a small quantity of uptake but only after the protoplasts were "aged" for 24 hours after isolation. They postulated that the exposure to cellulase and perhaps the osmotic shock of plasmolysis caused some membrane damage from which the protoplasts could at least partially recover after 24 hours. These explanations were substantiated by experiments in which maize leaves pre-loaded with ^{86}Rb were allowed to leak into a water control, 0.6M sorbitol, or 0.6M sorbitol with 2.5% cellulase. There was some leakage into the 0.6M sorbitol in the first hour, but this leakage did not increase significantly over the next 14.5 hours. There was considerably more leakage into the sorbitol and cellulase mixture which continued for the next 15.5 hours indicating that the cellulase damage exceeded that induced by osmotic shock. These results are not surprising in that Hall and Wood (40) have shown that cell wall degrading enzymes from soft rot parasites induce leakage long before the cells separate. However, there is another possible explanation for Taylor and Hall's (92) results in that some of the ^{86}Rb may have been released from binding sites on the cell wall upon degradation by cellulase.

It should be noted that Taylor and Hall's (92) low uptake rates may have been influenced by their experimental procedure: their protoplasts were subjected to millipore filtration which could have burst many of the delicate protoplasts. In addition, their lengthy isolation period (15 to 19 hours) subjects the protoplasts to the possible detrimental effects of the cellulase for far longer than the majority of isolation procedures.

Ruesink (81) studied the uptake of leucine by tissue culture cells and isolated protoplasts of Convolvulus. He found that 0.5M osmoticum inhibited uptake about 60% as compared with tissue in the absence of osmoticum. It made no difference whether the osmoticum was sucrose, mannitol, sorbitol, or ions, Uptake was further decreased by the addition of cellulase (in this case Ruesink's own Myrothecium preparation) in agreement with Taylor and Hall's (92) leakage data. Interestingly, boiling the enzyme preparation before exposing the tissue to it did not decrease the extent of inhibition, indicating that the inhibitory agent was some non-enzymatic toxin. In this regard, ribonuclease which was probably present in the preparation, may cause leakage via a charge interaction with the membrane in spite of inactivation of the enzymatic activity (80). The protoplasts, once separated from the enzyme mixture, regain some uptake capacity to the extent that they take up slightly more leucine than plasmolysed tissue in the presence of cellulase. However, they never achieved rates near those obtained with plasmolysed tissue in the absence of cellulase. Protoplasts from oat leaves, however, take up equally as much leucine as the plasmolysed tissue (85).

Robinson and Mayo (73) have recently reported an interesting study of the uptake of a number of compounds by cultured tobacco mesophyll protoplasts. The compounds tested were widely divergent, including leucine, uracil, glucose, phosphate, and mannitol. A common trend was noted in that protoplasts which had been cultured for 20-22 hours took up more of the tested compounds than protoplasts cultured for 1-3 hours.

This seems to agree with Taylor and Hall's concept of "aging" leading to repair and increased uptake capacity (92). In addition, they demonstrated that the uptake capacity was, to some extent, active, particularly in the case of protoplasts incubated for 20-22 hours. It was calculated that the protoplasts occupied about 0.1% of the volume used in the assay. Since the protoplasts took up greater than this proportion of all substances except mannitol, there was a concentration accumulation against a gradient and active uptake must have occurred.

3. Effects of Auxins. Protoplasts provide a potential to explore the mode of action of growth regulators. The potential is especially great with the auxins where the primary action is thought to involve an initial binding or interaction of the hormone with the plasmalemma (74). Power and Cocking (70) showed that IAA could burst all of the treated tobacco leaf protoplasts in four hours at the exceedingly high concentration of 10^{-3}M. In a slightly more physiological concentration range (10^{-4} to 10^{-5}M) eighteen hours were required. Ruesink and Thimann (77), however, found no effect on Avena coleoptile protoplasts at any concentration of IAA. Hall and Cocking (41, 42) reinvestigated this problem and discovered that at the concentration of mannitol used by Ruesink and Thimann (0.4M) there was no response to 10^{-3} to 10^{-4}M IAA. In addition, they observed 100% of treated protoplasts in 0.29M mannitol burst in five minutes when they were exposed to 10^{-5}M IAA. Control protoplasts in 0.29M mannitol were stable for at least two hours and exhibited only 2% bursting.

To substantiate this they were able to show a similar inhibition of growth by intact coleoptile sections exposed to IAA by 0.4M mannitol. Further substantiation comes from Pilet (66). He demonstrated that an increased sensitivity to auxin in mechanically isolated onion root protoplasts, relative to enzymatically isolated protoplasts, resulted from a higher osmotic pressure in the former such that they would be more inclined to burst in a particular concentration of mannitol than would the latter. He was also able to show a favorable comparison of response to IAA between the protoplasts and the intact roots from which they originated (65).

Bayer and Sonka (5) were able to show an instantaneous acidification with 10^{-4}M IAA with tobacco protoplasts indicating that the "lag phase" previously reported (74) may be an artifact of the experimental technique of using intact tissue. Some caution should be exercised in considering this data as no concentration curves were done and no auxin analogues were tested. More recently, the uptake of labeled IAA into protoplasts was demonstrated, but no time correlation between the uptake and acidification response could be shown (89). However, a correlation between ion uptake and the acidification response has been shown with protoplasts using the fungal toxin fusicoccin which has been shown to mimic auxin by bringing about proton extrusion (67).

It should be noted that IAA concentrations of 10^{-3} to 10^{-5}M have been used in all of the afore mentioned work with protoplasts. This is similar to the concentrations range that intact tissue is exogeneously exposed to in analogous studies. While those working with intact tissue may be able to justify their use of such high concentrations (relative

to physiological concentrations) in terms of diffusion problems, it is difficult to invoke this sort of explanation with protoplasts. The question, therefore, remains as to why plant cells and tissues must be exposed to such high concentrations of auxin to elicit a response.

B. Cell Wall Synthesis.

Ultrastructural studies on the formation of primary cell walls using intact plant tissues have provided limited information. Much more information can be gotten by studying wall formation with protoplasts where one is likely starting with the complete absence of a cell wall. Grout (38) examined cell wall formation by tobacco mesophyll protoplasts by deep etch electron microscopy. The first evidence of microfibril formation at the plasmalemma surface appeared 16 hours after the initiation of culture. He postulated that this period of time in culture is required for the recovery of the membrane and associated enzyme systems. By 72 hours in culture the surface shows the appearance of the primary cell wall. The wall at this point is the earliest stage of development seen when intact tissue is used.

A similar study was done by Burgess and Linstead (14) using scanning electron microscopy. They show fibrular development on tobacco protoplasts after 24 hours in culture. Unfortunately, their micrographs of freshly isolated protoplasts showed holes in the plasma membrane surface. Such holes have been attributed to certain fixation techniques used with scanning electron microscope (49). With this in mind, studies using these techniques should be compared carefully with others to try to eliminate interpretation based on artifacts.

C. Respiration and the Effects of Plasmolysis on Metabolism.

The concept that plasmolysis alters physiological responses arises frequently. Greenway (34) showed plasmolysis caused a reduction of respiration, decreased glucose uptake, and synthesis of methanol insoluble compounds by corn roots. Hoffman et al. (45) studied the respiratory rate of protoplasts isolated from petunia. They concluded that the low rates must be due to physiological damage incurred during isolation. Taylor and Hall (92) have data comparing respiration of leaf and root protoplasts to their respective tissues. They attribute this difference to the loss of non-respiratory protein (i.e. cell wall protein) in the case of the protoplasts resulting in higher rates when they are expressed on a protein basis. However, it was not clear whether or not the intact tissue was in plasmolyzing medium and it is possible that plasmolysis could lead to a decrease in respiratory rates (34).

While Greenway (34) demonstrated that osmotic stress resulted in a general decrease of metabolic activity, there are observations which suggest that there is a specific increase in the synthesis of ribonuclease in intact tobacco leaves (57) and isolated protoplasts (57, 71) due to osmotic stress. Ribonuclease activity was shown to increase 12 to 15 fold in both leaf discs and protoplasts in 0.7M mannitol over a period of 24 hours as compared to a control of leaves floated on water. This increase in activity was completely inhibited by cycloheximide and partially inhibited by kinetin which led to the suggestion that the increase was due to de novo synthesis of ribonuclease. The trigger

for this putative de novo synthesis was probably osmotic shock rather than physical damage incurred in protoplast isolation since the same effect was shown in intact tissue. However, the physical damage due to the rupture of plasmodesmata during plasmolysis was not considered in this early work (57). Recently (71) the possible effect of plasmodesmata rupture was examined and there was a low level of ribonuclease activity immediately after protoplast isolation indicating that the physical damage incurred during these procedures made a negligible contribution to the activity increase. The deleterious effect of osmotic shock was further characterized in an experiment in which various concentrations of different osmotica were tested for their effect on ribonuclease levels in leaf discs. Ethylene glycol, a rapidly permeating osmoticum, even at the concentration of 0.7M did not increase the ribonuclease level over the water control, while the very slowly permeating mannitol was increasingly deleterious at high concentrations. Greenway and Leahy (35) using corn root tips, also demonstrated a differential effect of rapidly and slowly penetrating osmotica. They concluded that rapid penetration minimized turgor loss and, therefore, decreased the adverse effects which could then be attributed to plasmolysis and water loss.

The extent to which protoplasts are altered in their metabolic competence due to the necessity of being maintained in a solution with a high osmotic concentration remains to be resolved.

D. Photosynthetic Studies.

1. C_3 Calvin Cycle Plants. Considerable variability has been reported in CO_2 fixation rates of protoplasts when compared to intact

tissue. Huber and Edwards (48) were able to achieve rates of 148 to 173 μmoles CO_2/mg chl x hr. using wheat protoplasts. These rates approach expected rates for C_3 calvin cycle plants at least as well as isolated Class I spinach chloroplasts (51). Nishimura and Akazawa (62) reported fixation of 33 to 75 μmoles of CO_2/mg chl x hr. using spinach protoplasts which means that the maximum rate was only half that of isolated spinach chloroplasts (51). Wegmann and Mulbach (98) got rates of 6 μmoles CO_2/mg chl x hr. using sunflower protoplasts. These rates are equally as poor as chloroplasts isolated from this tissue which *in vivo* fixes up to 300 μmoles CO_2/mg chl x hr.

2. C_4-Dicarboxylic Acid Pathway Plants. Interesting work is being done with photosynthesis in plants exhibiting the C_4-dicarboxylic acid pathway. The ability to separate mesophyll protoplasts from bundle sheath strands has resolved the controversy over the location of phosphoenolpyruvate carboxylase and clearly establishes the majority of the activity of this enzyme in the mesophyll cells (39, 48). In addition, bundle sheath cells have been shown to be capable of fixing CO_2 at a rate half that of the *in vivo* photosynthetic rate (52). Therefore, bundle sheath chloroplasts are somewhat autonomous in spite of the fact that they must obtain NADPH due to a deficiency in Photosystem I and CO_2 in the form of malate which is decarboxylated from the mesophyll cells. Mesophyll protoplasts from C_4 species achieve very little CO_2 fixation without the addition of phosphoenolpyruvate which leads to a ten-fold enhancement (39) resulting in rates which far exceed the *in vivo* rates of CO_2 fixation

for the intact tissue (52).

3. The Uptake of Chloroplasts to Increase Photosynthetic Efficiency. As mentioned above (sec. A. 1.), protoplasts can be induced to take up chloroplasts. Conceivably the transfer of chloroplasts from photosynthetically efficient species to less efficient species would result in increased plant productivity (15). Several successful transfers of chloroplasts have been reported. Carlson (15) and Potrykus (68) were able to transport wild type chloroplasts into albino mutants of Nicotiana tabaccum and Petunia hybrida, respectively. Carlson's work (46) however, is criticized because of lack of experimental detail. Furthermore, since Carlson was working with a vareigating albino, it may have been possible that his white individual was a periclinal chimera in which the plastids in the epidermis are genetically green but appear white because of their position in the epidermal cells. Such a white protoplast would be regenerated to a green plant without chloroplasts having been taken up (68). Bonnett and Eriksson (8) were able to make an interspecific transfer of algal chloroplasts into protoplasts obtained from carrot cell suspension cultures which are non-pigmented and cannot be induced to form chlorophyll or differentiate chloroplasts.

The uptake of chloroplasts does not appear to be spontaneous with the exception reported by Carlson (15). Common techniques for inducing uptake include the exposure to $NaNO_3$ during centrifugation (68) or exposure to polyethylene glycol (8). Even with these procedures the rate of uptake is quite low with only 0.5 to 1.0% of the chloroplasts entering protoplasts and 16% of the protoplasts receiving chloroplasts (68).

While techniques to increase the uptake of chloroplasts are being developed, there is some cause for question as to whether chloroplasts from a specific source can be made to function effectively in the cytoplasm of a plant of another species (32). It is entirely possible that the chloroplasts will survive and even reproduce for a period of time, as they have been demonstrated to do so in animal cytoplasm (31) and totally artificial environments (75). There is, however, evidence that chloroplasts subjected to interspecific transfer may not be able to synthesize ribulose 1,5-bisphosphate carboxylase which can catalyse CO_2 fixation. The evidence for this doubt comes from Criddle *et al.* (20) who have shown, using cycloheximide, that the small sub-units of the ribulose 1,5-bisphosphate carboxylase are synthesized in the cytoplasm and hence are likely coded for by nuclear DNA, while synthesis of the large sub-unit is more selectively inhibited by chloramphenicol, and therefore, is more likely synthesized in the chloroplast and coded for by chloroplast DNA. It may, therefore, be unreasonable to expect interspecific compatibility of large and small sub-units of ribulose 1,5-bisphosphate carboxylase leading to a functional enzyme. Hopefully it may be possible to carry out successful transfers of chloroplasts between members of closely related related species with some agronomic advantage in spite of these problems.

E. Nucleic Acid Metabolism.

Intact protoplasts carry on transcription to the extent that constant rates of incorporation of ^{14}C uracil can be observed once equilibration with the internal pools is complete (82). A comparison of

these rates to those obtained with intact tissue was not done.

Limited work has been reported regarding the capacity of higher plants to repair DNA damaged by ionizing or ultraviolet radiation. The use of protoplasts provides the ability to use plant cells much like microbes for this sort of work. Pyrimidine dimers induced in DNA by ultraviolet radiation can be repaired by protoplasts. The dark excision mechanism known to occur in animal cells and bacteria was first demonstrated in plants by the use of protoplasts while unsuccessful attempts were made using tissue culture (46). Howland et al. (47) were able to demonstrate that wild carrot protoplasts were able to repair 50% of DNA strand breaks after 20 k rads of γ radiation within five minutes. By one hour no breaks could be detected. These protoplasts, thereby, exhibit repair characteristics that are similar to all repair-competent microbial and animal cells (47).

F. Isolation of Organelles from Protoplasts.

Cell fractionation and purification of organelles has proved to be much more difficult for plant cells than animal cells because of the rigid cell wall. The grinding procedures required to break up the cell walls often damage the delicate organelles within, reducing yields and decreasing functional ability. Isolated protoplasts have provided the unique opportunity to obtain organelles without resorting to harsh grinding techniques.

1. Chloroplasts. Currently, Spinacea oleracea and Pisum sativum are the only species which provide isolated chloroplasts with envelope membranes intact enough to be used for CO_2 fixation (96).

Rathnam and Edwards (73) forced protoplasts through a 20 μm mesh which was of the appropriate size to break all of the protoplasts and allow the chloroplasts to go through without damage. Carbon dioxide fixation rates were comparable to those of intact protoplasts in all five species tested while the highest rates obtained by chloroplasts isolated by grinding was 40% that of the protoplasts.

2. Nuclei. Functioning nuclei can be isolated from protoplasts more readily than by grinding intact tissue. Blaschek et al. (7) were able to isolate from tobacco and petuna protoplasts a large number of nuclei which were able to carry out transcription at rates ten to one hundred fold higher than nuclei isolated by grinding.

3. Vacuoles. Perhaps one of the most amazing developments is the isolation of large numbers of intact vacuoles from protoplasts. Wagner and Siegelman obtained vacuoles from protoplasts from leaves, petals, and stems of many different plant species by osmotic shock in potassium phosphate buffer (95). Intact vacuoles were also isolated by Lörz et al. (58). They discovered that prolonged exposure to the cellulase resulted in displacement of the vacuole to one side of the protoplast. Taking advantage of this, they subjected the protoplast to centrifugal force in a sucrose gradient. This resulted in the disruption of many of the protoplasts and the isolation of vacuoles in the upper phase of the gradient. In this manner, vacuoles can be obtained from any tissue which will yield protoplasts. Insolated vacuoles will allow research on the permeability properties of the tonoplast and biochemical function of the vacuole, heretofore, not possible.

V. Conclusions.

The advent of procedures by which large populations of physiologically sound plant cell protoplasts can be obtained has given the plant scientist new opportunities in genetics and cell physiology.

In genetics the possibilities with plants are perhaps more far reaching than those with animals. Protoplasts can be cultured and fused in a manner similar to animal cells. In addition, protoplasts which have been cultured can be regenerated to whole plants.

Plant physiology was previously confined to the study of intact, excised tissues or crude homogonates. The use of protoplasts overcomes some of the inherent limitations of these techniques. The properties of the plasmalemma can be studied much more effectively than when intact, excised tissues are used. The ability to do ultrastructure studies of the protoplast surface has yielded information regarding the binding of various substances and pinocytotic uptake of large particles. In addition, similar techniques have been used to study the formation of the cell wall at the plasma membrane surface. The use of protoplasts for studies of metabolite uptake illiminates the problem of diffusion of substrate through cell layers and possible binding of substrate to cell walls. Other examples in which the use of protoplasts has expanded upon and clarified work done with intact tissue include the studies in which the location of carboxylases was established in plants with the C_4-dicarboxylic acid pathway of photosynthesis, the development of techniques for the isolation of intact and functional cellular organelles, and work regarding the plant's ability to repair DNA after ultraviolet or ionizing radiation.

The potential for basic studies in plant cell physiology using protoplasts are even greater than what has already been done. It is to be hoped that as these potentials become realized, the body of knowledge which accumulates may be applicable to the improvement of crop species.

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PART II

THE PHYSIOLOGICAL STATUS OF OAT LEAF PROTOPLASTS

The Physiological Status of Isolated Oat Leaf Protoplasts.

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SUMMARY

Isolated oat leaf protoplasts were compared to intact, excised leaves with regard to capacity to take up leucine, respire, and photosynthesize. In all cases, the protoplasts behave similarly to the intact leaves. One can therefore justify the use of protoplasts for physiological studies which cannot be done with intact tissue.

INTRODUCTION

Protoplasts have been regarded for some years as a valuable tool for somatic cell hybridization for plant breeding [1,2,3]. Only recently has interest in the use of protoplasts for plant cell physiology developed. Such diverse areas as the behavior of the plasma membrane [4,5,6,7,8,9], the nature of virus attack and replication [10,11], the action of growth regulators [12,13,14,15,16] , photosynthesis [17,18,19] and nucleic acid metabolism [20,21,22] have been studied with protoplasts from many species. However,little research exists to demonstrate the physiological competence of protoplasts as compared to intact tissue in spite of doubts expressed about this point[9]. With this in mind, the work described here seeks to demonstrate the physiological competence of protoplasts as compared to the leaves from which they were isolated. To this end, the photosynthetic, respiratory and uptake capability of oat protoplasts and oat leaf sections have been examined.

MATERIALS AND METHODS

Protoplast isolation.

Oats, Avena sativa L. cv Orbit (gift of Agway Seed Co.) were grown in vermiculite for seven to eight days in a growth chamber maintained at $23^{o} \pm 2^{o}$ with twelve hours of light (8.5 nE cm^{-2} sec^{-1}) from mixed fluorescent and incandescent bulbs. The seedlings were watered with full strength Hoagland's nutrient solution so that the vermiculite was kept continually moist.

One to two grams of tissue were prepared by stripping off the cuticle with electron microscopists forceps. The leaves were placed, peeled side down, on the surface of a digestion medium consisting of 0.6M sorbitol, 5mM $MgCl_2$, 5mM $CaCl_2$, 2mM dithiothreitol, 1% w/v glucose, and 20 mM MES adjusted to a final pH of 5.5 with KOH. This is a variation of the medium described by Kanai and Edwards[23]. The period of time (approx. 30 min.) during which the leaves were stripped and placed on the digestion medium serves to pre-plasmolyze the tissue. After the pre-plasmolysis period, cellulase (Cellulysin-Calbiochem.) in digestion medium is added to a final concentration of 0.5% w/v. After a two hour digestion period at 28^{o} to $30^{o}C$, the leaves were gently agitated to release the protoplasts. The protoplasts were pelleted by a low speed centrifugation (44 x g) in a clinical centrifuge and washed twice in a resuspension medium consisting of 0.6M sorbitol, 5mM $MgCl_2$, 5mM $CaCl_2$, 1% glucose w/v, and 20mM MES adjusted to a final pH of 5.5 with KOH. Two washes were sufficient to remove residual cellulase[24]. Further purification was accomplished when the protoplasts were allowed to settle out of a solution during a 30 to 45 minute period and supernatant containing the majority of

debris was removed. The protoplasts were then resuspended in fresh medium.

Measurement of chlorophyll.

Chlorophyll content of the protoplasts and leaves was determined by the method of Bruinisma [25]. Chlorophyll was used to quantify the material due to ease of measurement and greater reliability when compared to protein determination.

Measurement of leucine uptake.

Resuspension medium (pH 5.5) was used for all uptake experiments. [^{3}H] leucine (1 mCi/ml, 6 Ci/m mole) was added to 4 x 10^5 to 5 x 10^5 protoplasts to make up a total volume of 1 ml. The tubes containing the protoplasts were placed at a 45^{o}angle on a reciprocal shaker (80 cycles/min.) for various periods of time, or for 20 minutes when the leucine concentration was varied.

The uptake period was terminated by dilution with 10 ml. of ice cold resuspension medium. Collection of the protoplasts was by centrifugation at 44 x g for 15 minutes. This was followed by careful removal of the supernatant and resuspension of the protoplasts. Radioactivity associated with the protoplasts was determined with a Beckman model LS-100 scintillation counter with zero time points subtracted out to account for radioactivity associated with residual supernatant. Correction was made for the loss of counting efficiency due to quench by chlorophyll content. Aliquots of the samples were used to determine chlorophyll content and for hemocytometer counts of the remaining population.

The uptake of [^{3}H] leucine was determined for intact, excised leaves by floating sections from which the lower cuticle had been removed, peeled side down, on resuspension medium. Incubation periods and leucine concentrations were the same as those used for protoplasts. Termination of uptake was by aspiration of the radioactive medium followed by two five minute washes with ice cold resuspension medium. The sections were then aspirated to near dryness and put into scintillation fluid where they were kept overnight to dissolve before radioactivity was determined. Zero time points were subtracted out to account for radioactivity passively associated with the tissue. Correction was made for quench due to chlorophyll.

Respiration and photosynthesis studies.

Rates of respiratory oxygen consumption and photosynthetic oxygen evolution for both isolated protoplasts and excised leaf sections were measured with an oxygen electrode (Yellow Springs Instruments) at 28^{o}C. The population of protoplasts in suspension was adjusted so that the chlorophyll concentration was close to 0.1 mg/ml. as this was found to be optimal with regard to both photosynthesis and respiration. Measurements of respiration and photosynthesis were done both in resuspension medium and a photosynthetic medium for C_3 grasses [26]. Photosynthesis was initiated by the addition of $NaHCO_3$ (final conc. 6mM). Protoplasts and leaves received 60 nE cm^{-2} sec^{-1} of light (400-600 nm) from a photoflood lamp.

Carbon dioxide fixation for the protoplasts was determined simultaneously with O_2 evolution by labeling the $NaHCO_3$ to the extent of

4 Ci/ml with $NaH^{14}CO_3$ (Amersham-Searle). Twenty-five microliter aliquots were removed from the oxygen electrode chamber at various times and were acidified with ten microliters of 3N HCl. Carbon dioxide fixation was determined by liquid scintillation counting of acid stable products.

RESULTS AND DISCUSSION

Protoplast yields consitently averaged 8×10^6 to 10×10^6 cells per gram fresh weight of leaf tissue. This was similar to yields obtained by Brenneman and Galston[25]. It should be noted that these are high yields, especially for such a relatively short term isolation procedure. The chlorophyll content averaged 1.2 to 1.3 mg per 8×10^6 to 10×10^6 protoplasts. This represents about 50% of the chlorophyll content of a gram of tissue and is comparable to the values obtained by Huber and Edwards[26]using young wheat leaves. The preparation was reasonably free of chloroplasts and other cellular debris (fig. 1).

Leucine uptake.

The capacity to take up a metabolite was chosen as one of the parameters to be examined as this membrane associated capacity would likely be among the first to be damaged should the protoplasts suffer from the isolation procedure.

A time course for leucine uptake was done for leaves and protoplasts (fig. 2). Within the linear portion of the curve there was no significant difference in uptake between the protoplasts and leaf sections. In both cases there was a decrease in the rate of leucine accumulation between 40 and 60 minutes after the initiation of uptake. The decrease in rate occurs at a slightly lower level in the protoplasts.

It was first suspected that the rate was lowered due to a depletion of leucine. However, the use of a higher concentration of leucine (5 mM as opposed to carrier-free) showed the same pattern (data not shown) indicating sufficient substrate. Conceivably the protoplasts may be damaged during increasingly long uptake periods but this was not visibly evident as they maintained their spherical shape and polar displacement of the vacuoles did not increase with time. In addition, the decrease in rate cannot be the effect of the plasmolysing medium. The reasons are as follows: The decreased rates do not correspond with time in the presence of the plasmolysing medium as the time period differs for leaves and protoplasts but relates to time from the initiation of uptake. In addition, a similar time course was done for leaf tissue in non-plasmolysing medium (data not shown). The rates were lower for all time points and an even greater rate decrease was evident when compared to leaves in resuspension medium. Therefore, there is no obvious cause for the rate decrease.

The degree of uptake by the protoplasts was comparable to that observed by others [7,27] . For a 20 minute uptake period, there was excellent agreement of [^{3}H] leucine uptake shown here with the uptake of [^{3}H]α-amino isobutyric acid by similarly isolated oat leaf protoplasts. However, the uptake of the α-amino isobutyric acid was linear for greater that one hour [27]. Robinson and Mayo [7] have done leucine uptake experiments on cultured tobacco protoplasts. Calculations based on their results show that 50×10^{-6} p moles or leucine per hour were taken up by each cell. Freshly isolated oat leaf protoplasts take up 15.5×10^{-6} p moles of leucine per cell in one hour. The difference is slight and

may be accounted for by the possibility that tobacco protoplasts may be inherently more metabolically competent as evidenced by the ability to regenerate a cell wall.

Biphasic curves have been shown for the uptake of various concentrations of substrate, mostly with regard to the uptake of ions by intact, excised roots [28,29] . The resemblance to saturation kinetics common to enzymes has led to the postulation of membraneous carrier systems which differ in substrate affinity. If these kinetics could be shown for protoplasts as well as for intact tissue, the attribution of the effect to a membrane function is strengthened.

Concentration curves from 0.1mM to 5mM were done for both leaves (fig. 3) and protoplasts (fig. 4). There was a very slight suggestion of a biphasic curve for the leaves with the first "saturation" being in the region of 1mM. While this is the region for which Reinhold et al. [30] saturation with regard to α-amino isobutyric acid uptake by barley leaves, we do not feel that our evidence supports the concept of biphasic uptake for intact leaves. There is, however, a stronger suggestion of a biphasic curve with regard to the protoplasts with the first saturation occurring in the region of 1mM to 1.5mM.

The credibility of the biphasic curve for protoplasts is increased by determining how closely the first saturation approximates the rectangular hyperbola predicted by the Michaelis-Menten equation [31] . K_M and V_{max} were extropolated from the curve (fig. 4) and the data for the expected curve were calculated. While there was a slight significant deviation from the curve in the region of saturation, the data resemble the rectangular hyperbola described by the equation. While the results

are not conclusive, the suggestion of the biphasic curve by the protoplasts indicates that the phenomenon may be attributable to a membrane process.

Respiration and Photosynthesis.

While respiration and photosynthesis were initially studied in the resuspension and photosynthetic media, most of the protoplasts were found to break upon agitation in the lower osmoticum of the photosynthetic medium. Therefore, even though similar rates of respiration and O_2 evolution were exhibited by protoplasts and leaves in both media, data from experiments in resuspension medium alone are reported.

For protoplasts, rates of respiratory O_2 consumption averaged 6.13 μmoles O_2/mg. chl. x hr. (Table I) with values as high as 8.90 μmoles O_2/mg. chl. x hr. observed. Photosynthetic O_2 evolution showed a range of values, averaging 28.60 μmoles O_2/mg chl. x hr. with rates occasionally reaching 40 or 50 μmoles/mg. chl. x hr..

The protoplast population which was optimal had a chlorophyll concentration of 0.1 mg/ml as shown in the concentration curve (fig. 5). While the decline in the photosynthetic rate associated with the increased concentration may be due to self-shading, there is no obvious explanation for the lower rates of respiration at the higher concentrations of protoplasts or decreases at the very low concentrations.

When the respiratory and photosynthetic rates of protoplasts and leaf sections are compared (Table I) rates for protoplasts tend, on the average to be higher than those for leaves. In both cases the

rates of respiration fall within those expected for green leaf tissue (4-30μmoles O_2/mg. chl. x hr.) [32] . Similarly, higher respiratory rates were observed for maize protoplasts compared to maize leaf tissue by Taylor and Hall [9] . They attribute the slightly higher rates observed for the protoplasts to the loss of non-respiratory protein i.e. cell wall protein. If this were the case, one would not expect this sort of trend when data are expressed on a chlorophyll basis such as ours are. Such small differences may best be attributed to a more rapid and efficient diffusion of gases when one is dealing with isolated protoplasts as opposed to leaf sections.

Protoplasts were kept overnight in a Petri plate in the refrigerator to minimize bacterial growth, and rates of respiration and O_2 evolution were measured 24 hours after isolation. The initial purpose was to determine if they would remain viable over this time period. In all cases, rates were even higher for the "aged" protoplasts. The magnitude of this increase was generally 50% to 60% over that of freshly isolated protoplasts. The increased rates could arise from a growing bacterial population in a non-sterile protoplast preparation. However, it can be argued that while this is conceivable for respiratory increases it is difficult to attribute the concomitant increase in oxygen evolution (and CO_2 fixation-not shown) to bacteria. Such increases can best be attributed to a "recovery" from isolation or an otherwise developing increasing permeability of the plasmalemma to the gases exchanged. A similar recovery with aging was shown with an increased uptake of ^{86}Rb by maize protoplasts after they were stored for 24 hours [9]. The effect of aging on the uptake of various metabolites by cultured

tobacco protoplasts may also be similar [7] . For example, there was close to a 90% increase in the uptake of leucine by protoplasts cultured for 20 to 22 hours, as compared to protoplasts cultured for 1 to 3 hours.

The ability of protoplasts to fix carbon dioxide was measured concurrently with oxygen evolution by initiating the oxygen evolution with $NaH^{14}CO_3$. Carbon dioxide fixation was measured for 20 minutes and was found to be linear for that period of time regardless of rate. Fixation rates averaged 51μmoles CO_2/mg. chl. x hr. and were occasionally as high as 97μmoles CO_2/mg. chl. x hr., approaching rates expected for intact C_3 plants [33]. The ratio of O_2 evolved to CO_2 fixed ranged between 0.47 to 0.73, indicating that between 1 and 2 molecules of CO_2 were fixed per molecule of O_2 evolved. This only approaches the theoretical 1 to 1 ratio expected. Carbon dioxide fixation rates in the dark (data not shown) were not high enough to support the argument that CO_2 fixation might initially be supported by ATP and NADPh transported indirectly from the cytoplasm [34]. If energy and reducing power had been stored in the chloroplast during the hours in dim light during isolation, then rates of O_2 evolution, which must be measured in the first 5 minutes after the addition of the $NaHCO_3$, might be underestimated and would not compare favorably with CO_2 fixation. The problem of the discrepancy between the expected ratio and that obtained from the data remains unresolved.

Oat leaf protoplasts have been shown to behave similarly to intact leaves from which they originate. Therefore, while oat proto-

plasts have been refractory to growth in culture [24] , the difficulty is probably not due to the physiological status of the protoplasts, at least in so far as can be detected by these techniques. Furthermore, protoplasts which exhibit similarity to intact tissue with respect to parameters which are easily measured in both can justifiably be used for studies which cannot be adequately done with intact tissue. Protoplasts, thereby, provide a simpler system in which to study plant cell physiology.

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FIGURE LEGENDS

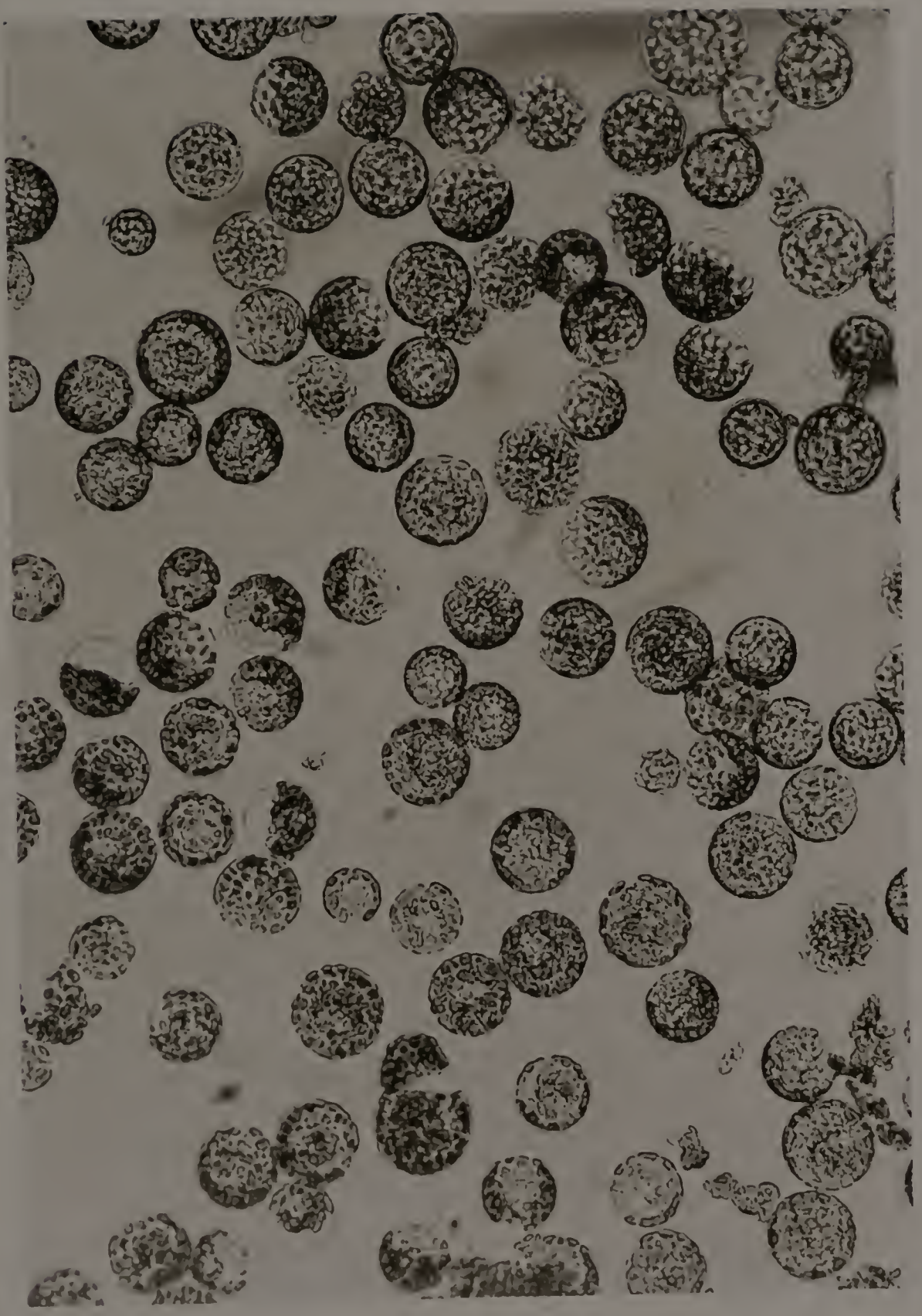
Figure 1. Protoplast preparation. x 160

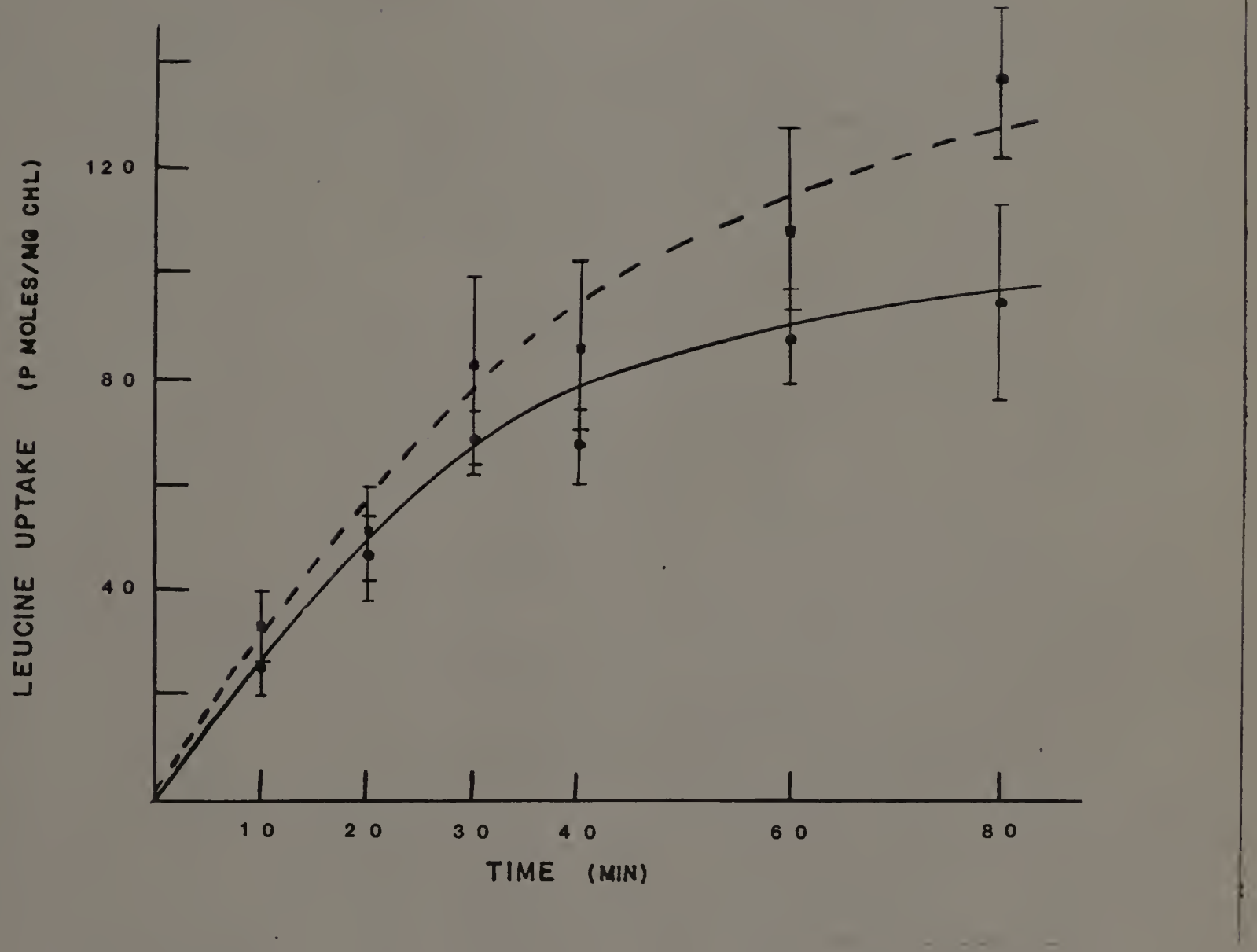
Figure 2. Time course for the uptake of leucine (carrier-free) by leaves (■) and protoplasts (●). Average of three experiments.

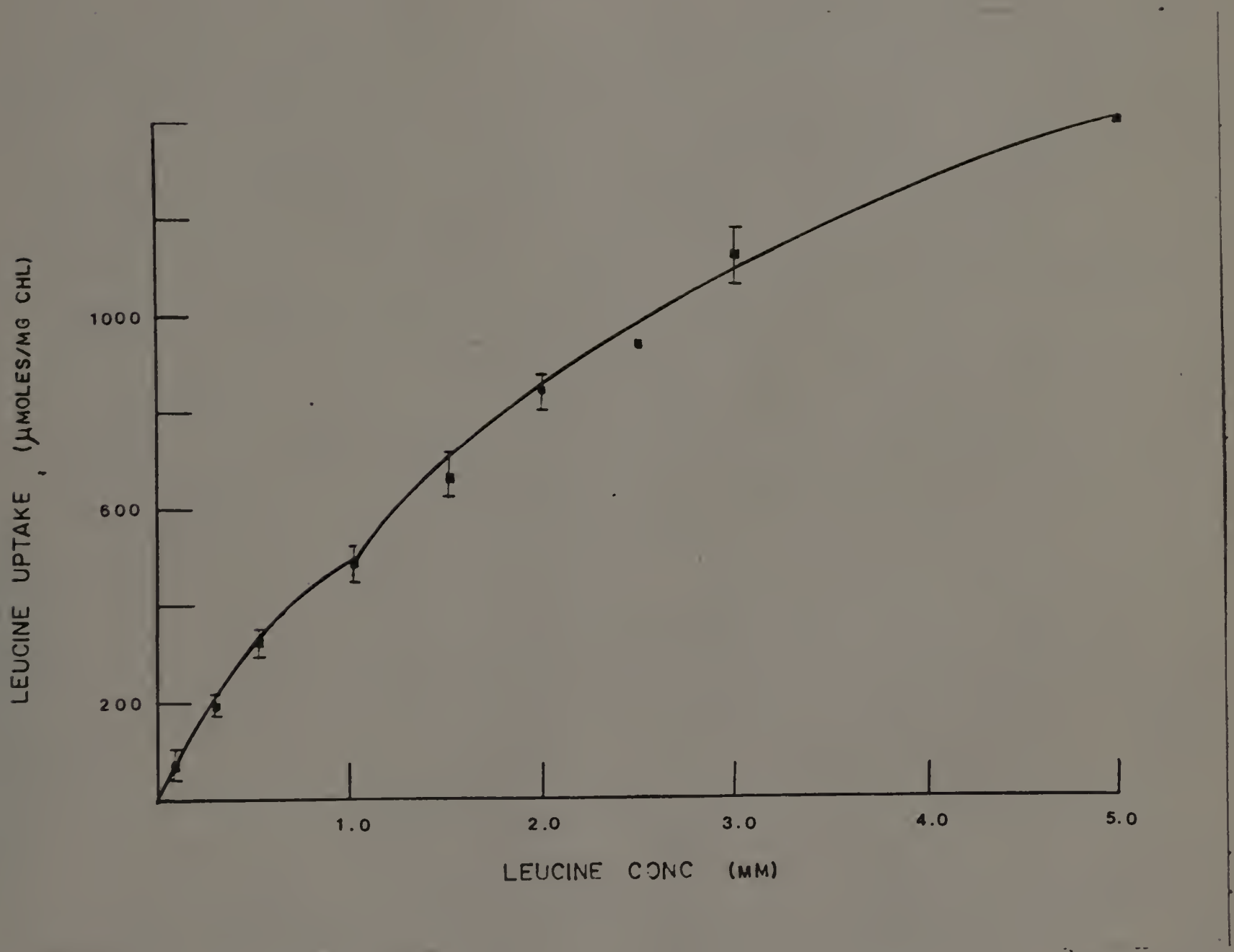
Figure 3. Concentration curve of leucing uptake by leaves. Representative data of an experiment done three times.

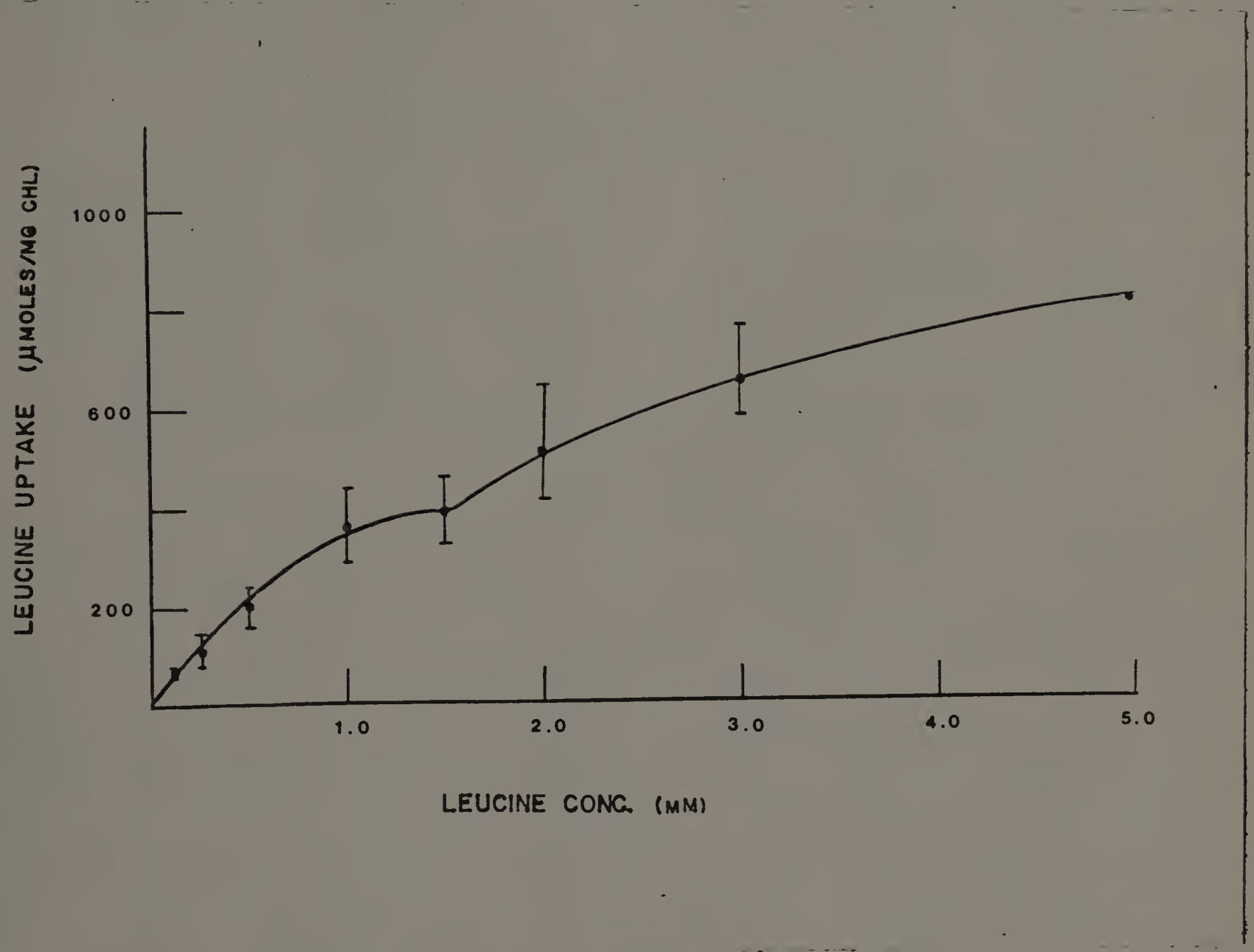
Figure 4. Concentration curve of leucine uptake by protoplasts. Data are representative of three experiments.

Figure 5. Chlorophyll concentration curve for the measurement of respiration and oxygen evolution by the oxygen electrode. Results of one of two experiments shown.




LEUCINE UPTAKE (P MOLES/MG CHL)
120
80
40
10
20
30
40
60
80
TIME (MIN)


LEUCINE UPTAKE (µMOLES/MG CHL)
1000
600
200
1.0
2.0
3.0
4.0
5.0
LEUCINE CONC (MM)


LEUCINE UPTAKE (μMOLES/MG CHL)
1000
600
200
1.0
2.0
3.0
4.0
5.0
LEUCINE CONC. (MM)

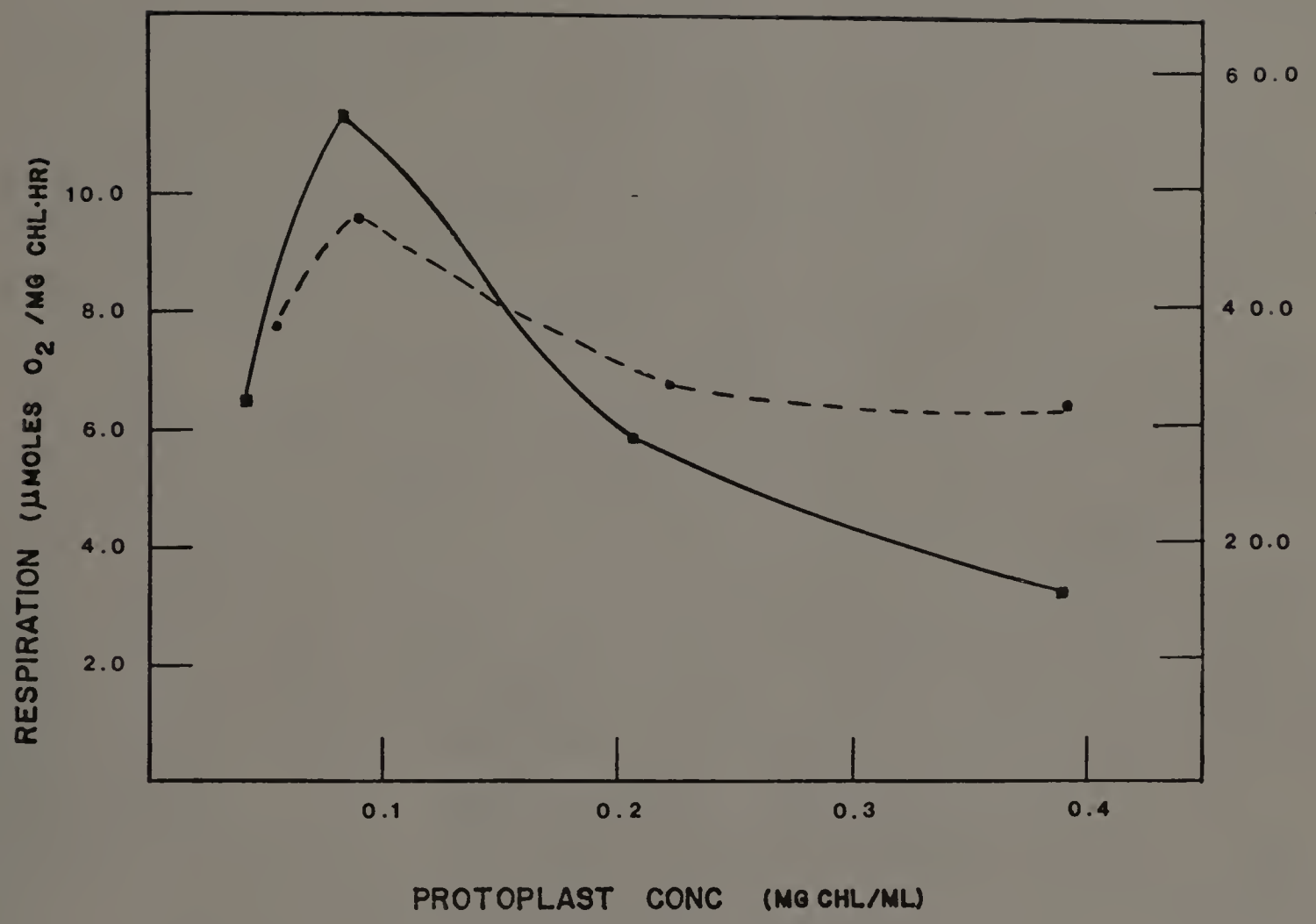

RESPIRATION (µMOLES O2 /MG CHL·HR)
10.0
8.0
6.0
4.0
2.0
60.0
40.0
20.0
PHOTOSYNTHESIS (µMOLES O2 /MG CHL·HR)
0.1
0.2
0.3
0.4
PROTOPLAST CONC (MG CHL/ML)

TABLE II

EFFECT OF AGING ON PROTOPLAST RESPIRATION AND O_2 EVOLUTION

Respiratory O_2 consumption μ moles/mg chl.hr.		Photosynthetic O_2 evolution μ moles/mg ch.hr.	
Fresh	Aged	Fresh	Aged
4.81	8.91	16.45	32.44
5.00	8.36	18.06	25.61
4.39	7.13	15.76	41.92

Experiments were done in resuspension medium. Fresh protoplasts were used within one hour after isolation was complete. Aged protoplasts were kept under refrigeration in a petri plate for 24 hours.

TABLE I

RESPIRATION AND PHOTOSYNTHESIS-LEAVES AND PROTOPLASTS

Respiratory O_2 consumption μ moles/mg chl.hr.		Photosynthetic O_2 evolution μ moles/mg chl.hr.	
Leaves	Protoplasts	Leaves	Protoplasts
5.73	6.13	26.15	28.60

Experiments done on fresh protoplasts and leaves at 28^{o} in resuspension medium. Protoplast chl. concentration approx. 0.2 mg in 2.0 ml. Leaf sections of 0.03 g representing 0.06-0.08 mg. of chl. were used.

Protoplast respiration and photosynthesis values are the average of 15 experiments. Leaf respiration values are the average of 7 experiments and photosynthesis the average of 4 experiments.

TABLE III

PROTOPLAST O_2 EVOLUTION AND CO_2 FIXATION

O_2 evolution μmoles/mg chl.hr.	CO_2 fixation μmoles/mg chl. hr.
28.22	50.94

Rates of O_2 evolution and CO_2 fixation were measured simultaneously (see materials and methods). Values are the averages of 7 experiments.

PART III

APPENDIX

OTHER PROTOPLAST ISOLATION PROCEDURES TRIED

1. Tissue: Corn coleoptile

Procedure: -strip epidermis and cut small subapical sections

-place sections in 50 µl of 1.0 M mannitol pH 6.8 (pH arbitrary)

-add 50 µl of 0.5% Cellulysin in 1.0M mannitol with 5 mM $MgCl_2$

-one hour and overnight incubations in the dark were terminated by the addition of 2.0 ml or 0.5 M mannitol.

-allow protoplasts to settle then resuspend, repeat to wash.

Variations: -incubate at 30^{o} in the waterbath for 3 hrs. and 26 hrs.

-0.5% and 2% Cellulysin used.

-tried procedure with leaf from within coleoptile and root.

Results: -protoplasts were never obtained within 2-3 hr. incubations. When left overnight (incubation periods approx. 15-26 hrs.) few protoplasts were obtained with the majority of the tissue being in the form of partly digested debris.

References: -Ruesink, A. W. and K. V. Thimann (1965). Protoplasts from the avena coleoptile. Proc. Nat. Acad. Sci. USA. 54:56-64.

2. Tissue: Corn coleoptile, bean leaves.

Procedure: -cut tissue into 1.0 ml of solution containing 0.8 M mannitol with 0.5% Macerozyme, 4.0% Cellulysin, pH 5.5

-incubate 2-3 hrs. at 25^{o} in a shaking water bath.

-terminate incubation by filtration.

-centrifuge 1 min. at low speed in IEC clinical centrifuge.

-resuspend in solution without enzyme.

Results: -in all cases very few poorly formed protoplasts.

References: -a representative sample of a number of references used to synthesize the above procedure.

-Power, J. B. and E. C. Cocking. 1970. Isolation of leaf protoplasts:macromolecule uptake and growth substance response. J. Exptl. Bot. 21:64-70.

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3. Tissue: Bean leaves, oat leaves.

Procedure: -cut 1.5 g of 13 day old leaves.

-carry out 15 min. preplasmolysis on digestion medium (dm) containing 0.6M sorbitol, 20mM MES-KOH, pH 5.5, 5mM $MgCl_2$, 1% sucrose and 2mM dithiothreitol.

-add more dm with dissolved Cellulysin such that the final concentration of cellulase is 0.5%.

-incubate 3 hrs. with stirring every 15 min., try overnight.

-terminate incubation by filter through two layers of cheesecloth and wash leaves twice with 10 ml of dm.

-centrifuge the filtrate at 400 x g for 3 min. and resuspend the pellet in a resuspension medium consisting of 0.6M sorbitol, 50mM Hepes-KOH, pH 8.0, 5mM $MgCl_2$, and 1% sucrose.

Results: -Bean leaves yielded negligible quantities of protoplasts while the oat leaves provided the first protoplast preparation of any quantity. It was, therefore, decided to continue the work with oat leaves.

References: -Peter Conrad, personal communication.

-Kanai, R. and G. E. Edwards. 1973. Purification of enzymatically isolated protoplasts. Plant Physiol. 52:484-490.

DETAILED PROTOPLAST ISOLATION PROCEDURE

1. Peel the cuticle off the leaves by pinching the area near the apex with the curved portion of an electron microscopists' forceps. Cut off the bruised portion and use only the mid-section of the leaf which should be most completely peeled.

2. Immediately place each leaf section, peeled side down, on 15 ml of digestion medium (see materials and methods) which is in a 15 in diameter petri plate kept on ice during peeling.

3. Dissolve 0.15 g of Cellulysin in an additional 15 ml of digestion medium which gives 0.5% cellulase when added to the leaves.

4. Put the petri plate in a water bath kept at 28-30^{o}C. for 2 hr.

5. Release the protoplasts by agitation with a broken off pasteur pipette. Do not force the leaves apart as this will result in greater quantities of debris. Well digested leaves will become darker in color. It should be noted that basal portions of the leaf section may not digest as completely and care should be taken to omit these portions of the leaf section when digestion is incomplete.

6. Once the protoplasts are released, tilt the petri plate and push the remainder of the leaves away from the liquid. Slowly take up the protoplasts with the broken off pasteur pipette (the pipette should be broken off so that the protoplasts are not forced through a narrow tube which would exert shear forces).

7. Protoplasts are deposited in tubes which fit in the IEC clinical centrifuge. Once the first protoplasts are in the tube, subsequent additions should be made by placing the tip of the pipette below the

surface of the liquid and slowly ejecting the protoplasts.

8. Centrifuge at 200 RPM (≈44 x g) for 15 min.

9. Pour off the supernatant and slowly add 10 ml of resuspension medium (see Materials and Methods). Resuspend by slowly pulling up into and ejecting from the broken off pasteur pipette. Centrifuge as before and follow with another wash.

10. Resuspend the protoplasts from 1 g of tissue to 2 or 3 ml with resuspension medium and allow to sit until the majority of protoplasts have settled out and the solution is a uniform light green. Remove the majority of the supernatant--carefully and resuspend. See explanation of Purification procedure following. The protoplasts are now ready for whatever...

Note: This procedure is a variation of the procedure described in "Other Protoplast Isolation Procedures Tried #3." Variations were partly a result of personal communication with Dr. R. Kaur-Sawhney and my own experience. A 70% increase in yield over procedure #3 is achieved with this procedure.

EXPERIMENTS IN OAT LEAF PROTOPLAST PURIFICATION

Due to the presence of free chloroplasts and some undigested tissue debris which sedimented with the protoplasts, an additional purification beyond the washes was sought.

1. Two-phase polyethylene glycol-dextran gradient described by Kanai, R. and G. E. Edwards, 1973. Purification of enzymatically isolated mesophyll protoplasts from C_3, C_4, and CAM plants using an aqueous dextran-polyethylene glycol two-phase system. Plant Physiol. 52:484-490.

a) Attempts to adapt the gradient to large protoplast populations and assessment of capacity to retain the maximum number of protoplasts.

1. Crude preparation-8,666,666 protoplasts/gm.fr. wt.

Dilution protoplasts from 1 g to	Expected 100% yield/ 0.6 ml.	$ Recovery	No. gradients required
2.0 ml.	3,900,000	41.7	3
3.0 ml.	2,666,800	62.1	5
4.0 ml.	2,011,500	46.3	8

2. Crude preparation-7,480,000 protoplasts/gm fr. wt.

2.0 ml.	2,244,000	52.0	3
3.0 ml.	1,320.000	71.8	5
4.0 ml.	1,200,000	50.5	8

These results indicate that the maximum recovery can be gotten from the gradient when protoplasts from 1 gram of tissue are suspended to 3.0 ml. However, 5 gradients would be required for each preparation and this is somewhat impractical.

b) Determination of the effectiveness of the gradient in removing free chloroplast debris.

The nature of two-phase polymer gradients is such that as the mixed components are being centrifuged an interface forms between the polyethylene glycol and dextran. The larger particles have a greater tendency to go to the interface and the smaller particles would accumulate elsewhere. The extent to which this tendency operates depends on, in addition to the physical nature of the polymers, charges within the gradient. Charge, therefore, is one parameter which can be altered to achieve a specific effect with the gradient. This can be done by varying the concentration of the $NaPO_4$ buffer.

Reference: Albertsson, P.A. 1971. Partition of Cell Particles and Macromolecules. Wiley-Interscience.

A comparison of protoplast recovery and free chloroplast removal with varied concentrations of $NaPO_4$ buffer and substituted HEPES buffer was done (representative of two trials).

Crude-9,492,000 protoplasts 20,250,000 free chloroplasts

buffer	% recovery protoplasts	% free chloroplasts remaining
2.5mM $NaPO_4$,pH 7.5	58	73
1.0mM $NaPO_4$,pH 7.5	48	55
2.0mM $NaPO_4$,pH 7.5	50	100
2.5mM HEPES,pH 7.5	53	49

It was decided that the gradient was inadequate in that there would be a loss of approximately 50% of the protoplasts to remove at the most 50% of the chloroplast debris.

2. Continuous dextran gradient: variations tried:

a) 10% w/w dextran in 0.6M sorbitol-25% w/w dextran in sorbitol 300 x g centrifugation for varied time periods. Band of protoplasts moved down in the gradient with no separation.

b) 5% w/w dextran in 0.6M sorbitol-10% dextran in sorbitol. Protoplast band underlayed by a group of clumped protoplasts.

c) 0.6M sorbitol-10% dextran in 0.6M sorbitol. Protoplasts spread throughout the gradient.

Since there was no rational conclusion or direction one could take from these results, the possibility of using the continuous gradient was discarded.

3. Wash by allowing the protoplasts to settle out of solution, removal of the supernatant, and resuspension.

a) Varied times of settling out, quantification of protoplasts retained and chloroplasts removed.

min. settling out of 3.0 ml. of soln.	% remaining in pellet compared to crude prep.	
	protoplasts	chloroplasts
30	84.7	34.9
	98.5	39.7
45	89.5	39.1
	99.2	47.0
60	87.8	45.2
	94.7	38.9
90	87.9	56.5

The time optimum was taken to be 45-60 minutes when protoplasts are allowed to settle out of 3.0 ml. of resuspension medium. Later, it was noticed that a good way to guage the time requirement of individual preparations is to remove the supernatant when it is a uniform shade of light green and a distinct separation from the pellet is evident.

A disadvantage of this purification technique is that the quality of the crude preparation is reflected in the final cleaned prep. ie. if the preparation has a lot of debris due to poor digestion a greater proportion of debris will be found in the pellet as compared to a good initial preparation. The advantage is that it is an adequate method of purification which does not subject the protoplasts to high molecular weight polymers whose effects on membranes and metabolism are unknown.

ATTEMPTS TO USE A FLOW DIALYSIS SYSTEM FOR UPTAKE EXPERIMENTS

A flow dialysis chamber was set up with a fraction collector in order to do uptake experiments in a similar manner to work done by Kaback with bacterial vessicles.

The system has the apparent advantage that none of the material undergoing uptake is lost as with the centrifugation and resuspension techniques, and one can tell what state the protoplasts are in afterwards as opposed to the millipore filtration technique.

Preliminary experiments indicated no effect on the level of radioactive leucine in the effluent by the presence of protoplasts on the other side of the dialysis membrane. Techniques for keeping both chambers agitated and aeration of the resevoir containing labeled substrate were all tried. Finally it was discovered that an insufficient portion of the label was able to pass through the dialysis membrane within a reasonable period of time. This was shown by loading the upper chamber with labeled leucine in resuspension medium and passing resuspension medium with no leucine through the lower chamber. Radioactivity in the upper chamber was measured before and after the run. The data are as follows:

1) Counts from upper chamber

initial	1,430,410	
final	- 644,390	
	686,020	CPM lost, therefore 49% of the counts went through the membrane in 40 ml. approx. 4 hrs.

2) Counts from upper chamber

initial	1,380,390
final	- 460,837
	919,553 CPM lost, 66% of the counts went through the membrane in 60 ml.

It was evident from these experiments that it would take many hours for minute quantities of label to go across the membrane and at no particular time would there be enough leucine in the upper chamber that uptake by protoplasts could be reflected in the effluent. It was decided at this point to use a centrifugation and resuspension technique (described in Part II) as along with the measurement of uptake there can be quantification of the remaining protoplasts in terms of chlorophyll content and actual cell counts.